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Kaigala et al.

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(54) **SUBTRACTIVE MICROFABRICATION AND FUNCTIONALIZATION OF SUBSTRATES BY HYDRODYNAMIC FLOW CONFINEMENTS**

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B01L 3/00 (2006.01)

(52) **U.S. Cl.**
CPC . ***B01L 3/502707*** (2013.01); *B01L 2200/0684* (2013.01); *B01L 2200/12* (2013.01); *B01L 2300/088* (2013.01); *B01L 2400/0463* (2013.01)

(58) **Field of Classification Search**
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See application file for complete search history.

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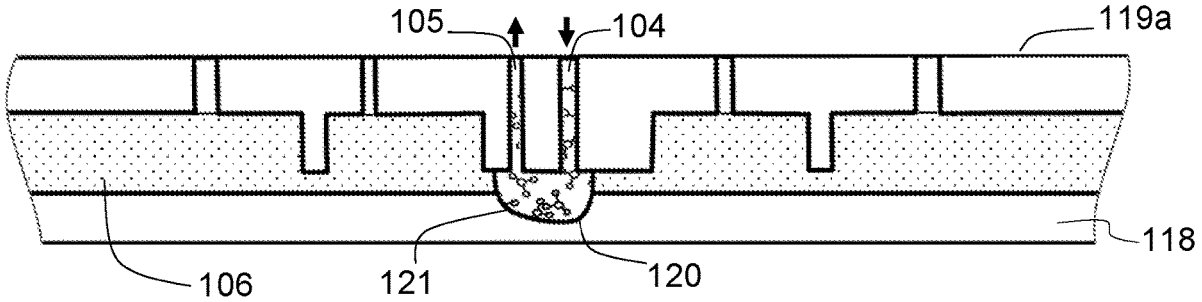
(57) **ABSTRACT**

Patterning a substrate can be provided. A substrate is covered by an immersion liquid and a microfluidic probe head is positioned in proximity with the surface of the substrate, so as to immerse a processing surface of the probe head in the immersion liquid. Liquid flows are generated between the processing surface of the probe head and the surface of the substrate, via the probe head. The liquid flows generated include an etching flow of an etching liquid (e.g., an acid or solvent) and a processing flow of a processing liquid (e.g., a solution or suspension).

17 Claims, 6 Drawing Sheets



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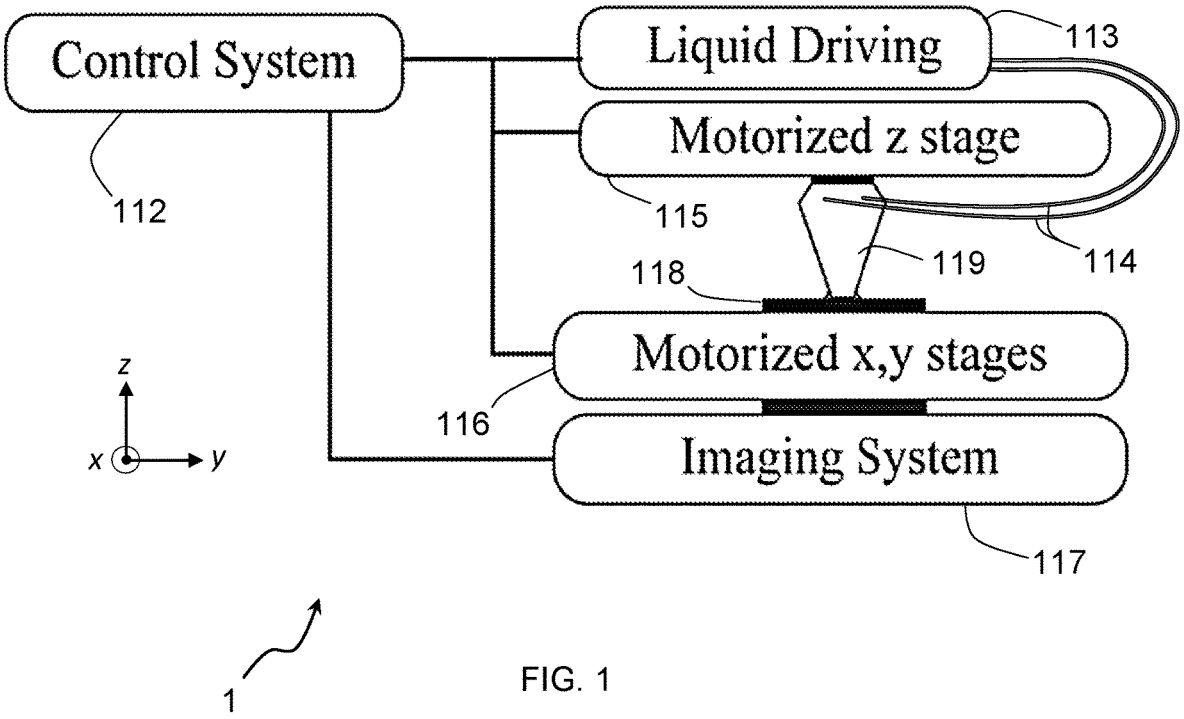
Control System
112
Liquid Driving
113
Motorized z stage
115
114
119
118
Motorized x,y stages
116
Imaging System
117
z
x
y
1

FIG. 1

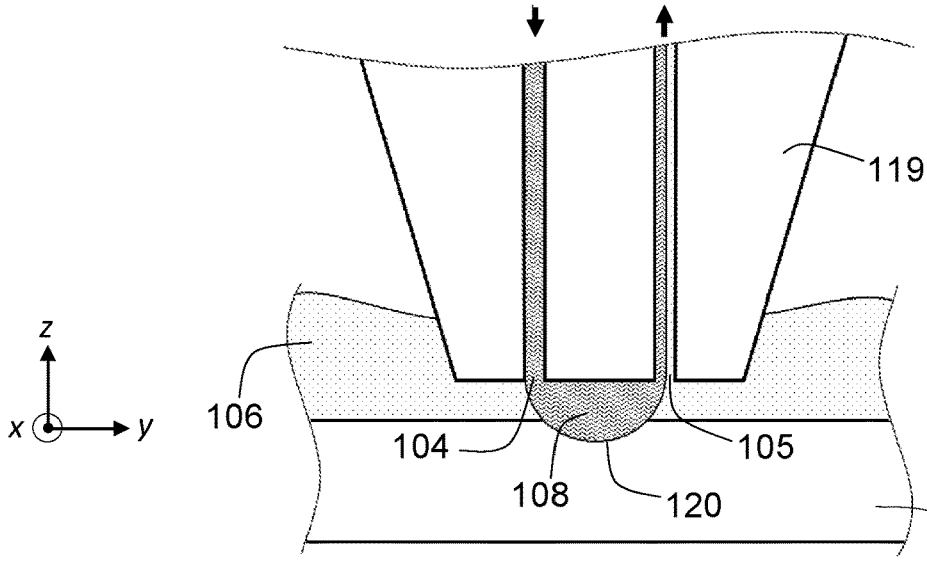

FIG. 2A

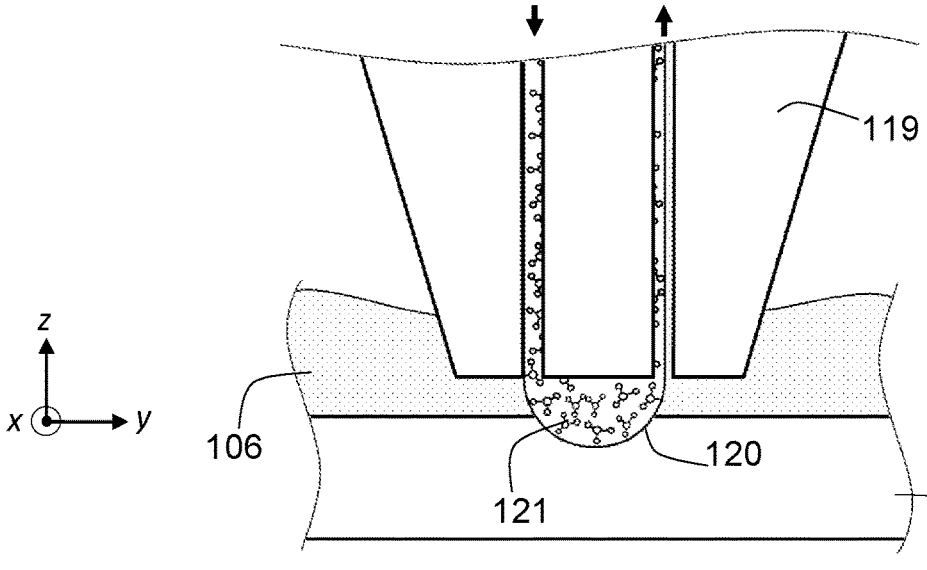

FIG. 2B

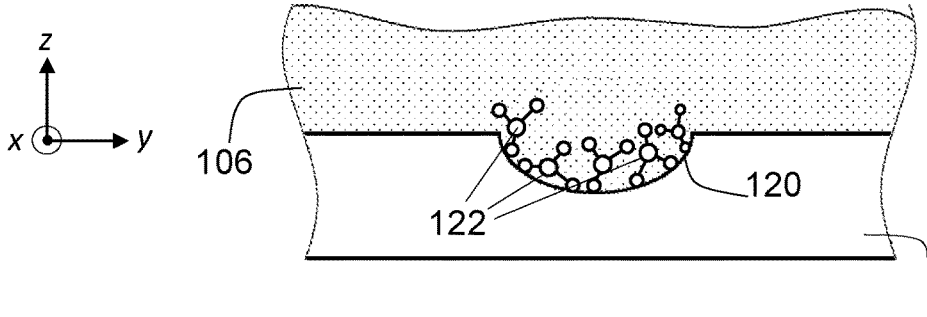

FIG 2C

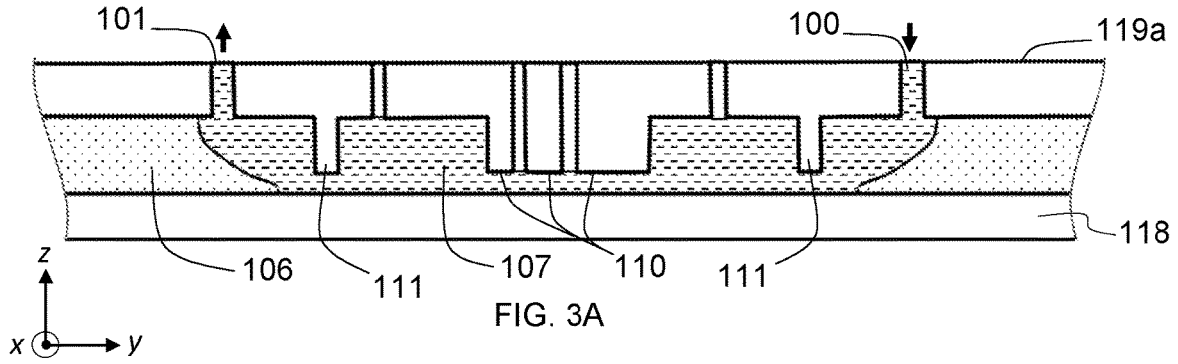

FIG. 3A

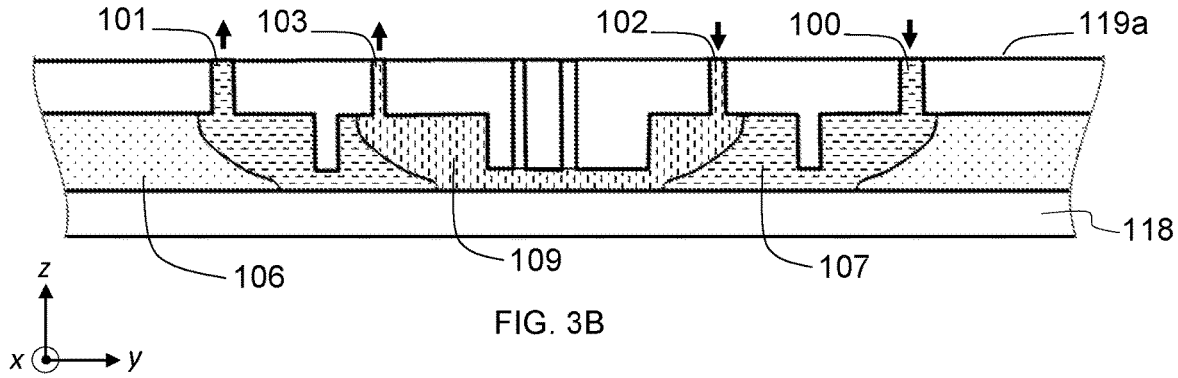

FIG. 3B

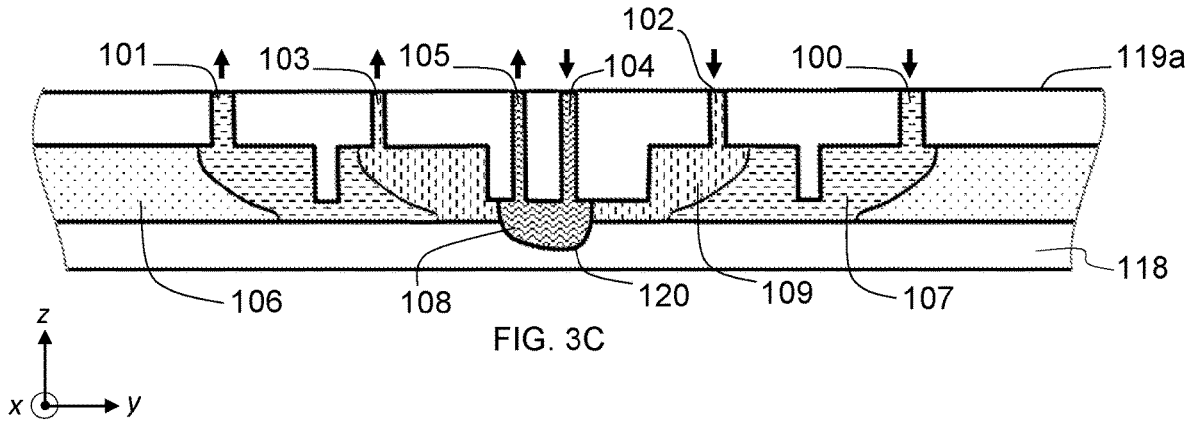

FIG. 3C

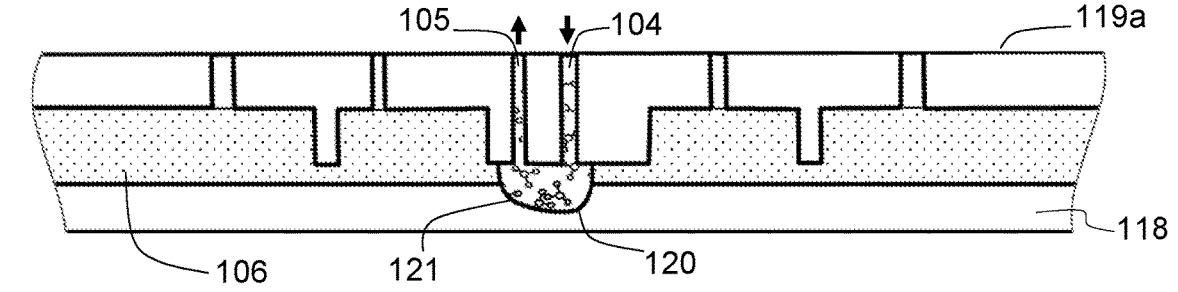

FIG. 3D

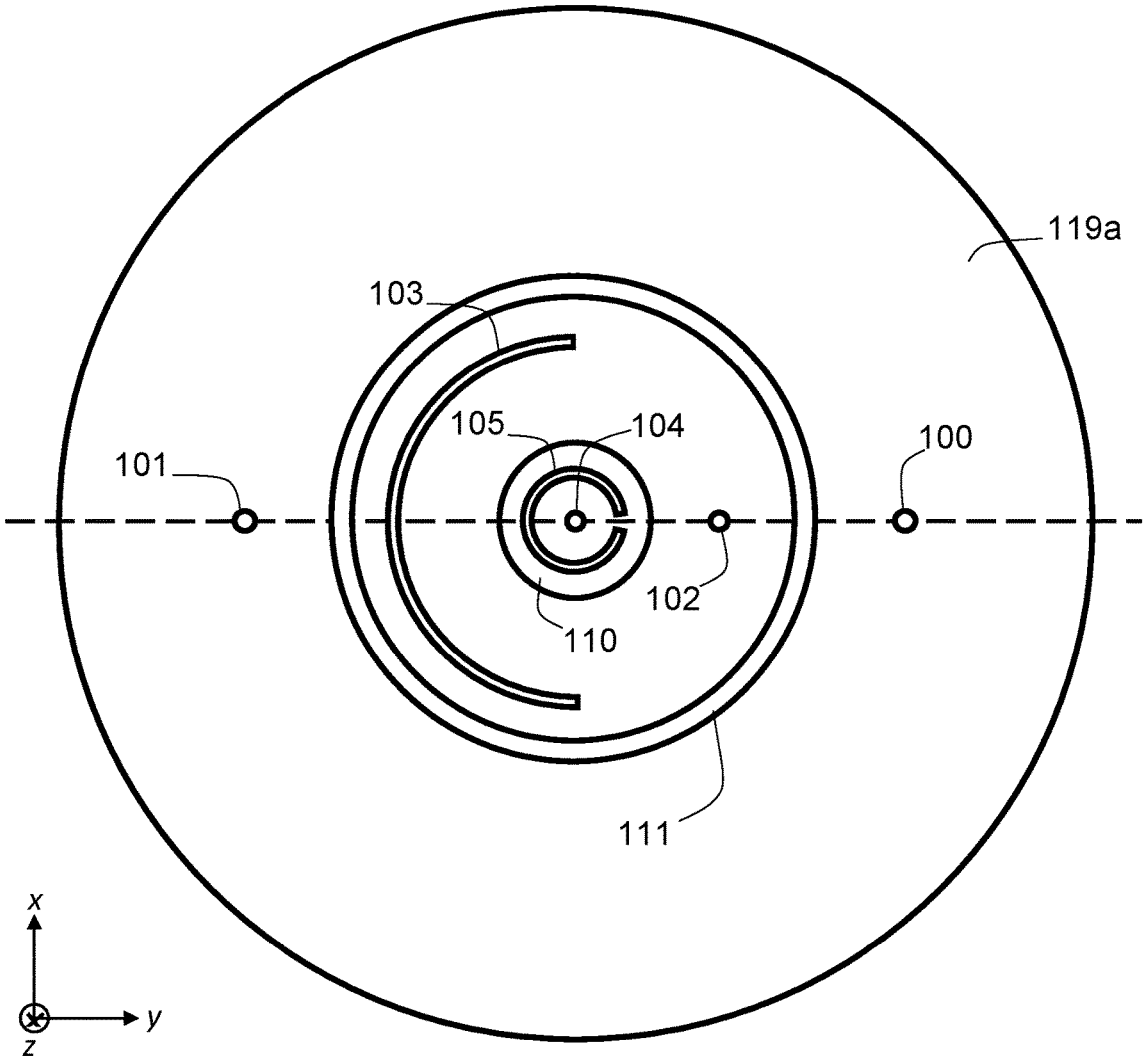
119a
103
105
104
101
100
102
110
111
x
y
z

FIG. 4

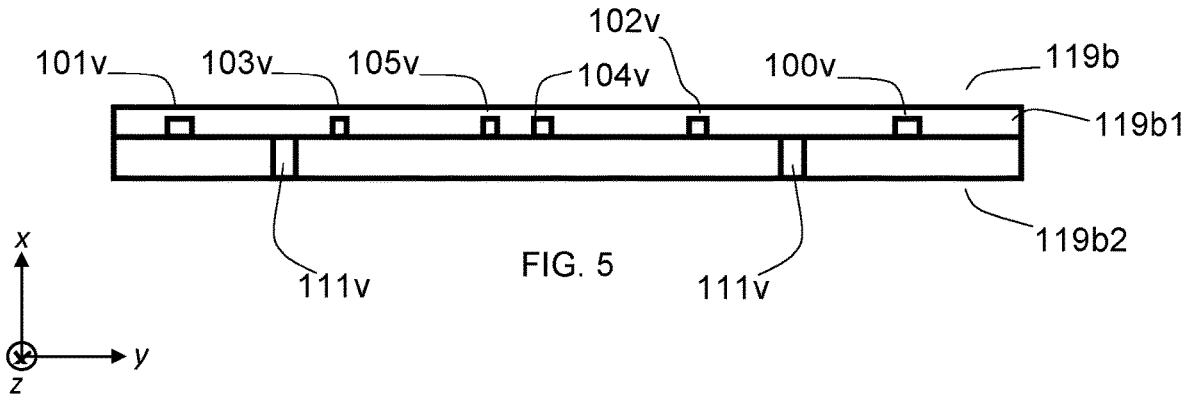
102v
101v
103v
105v
104v
100v
119b
119b1
119b2
111v
111v
x
y
z

FIG. 5

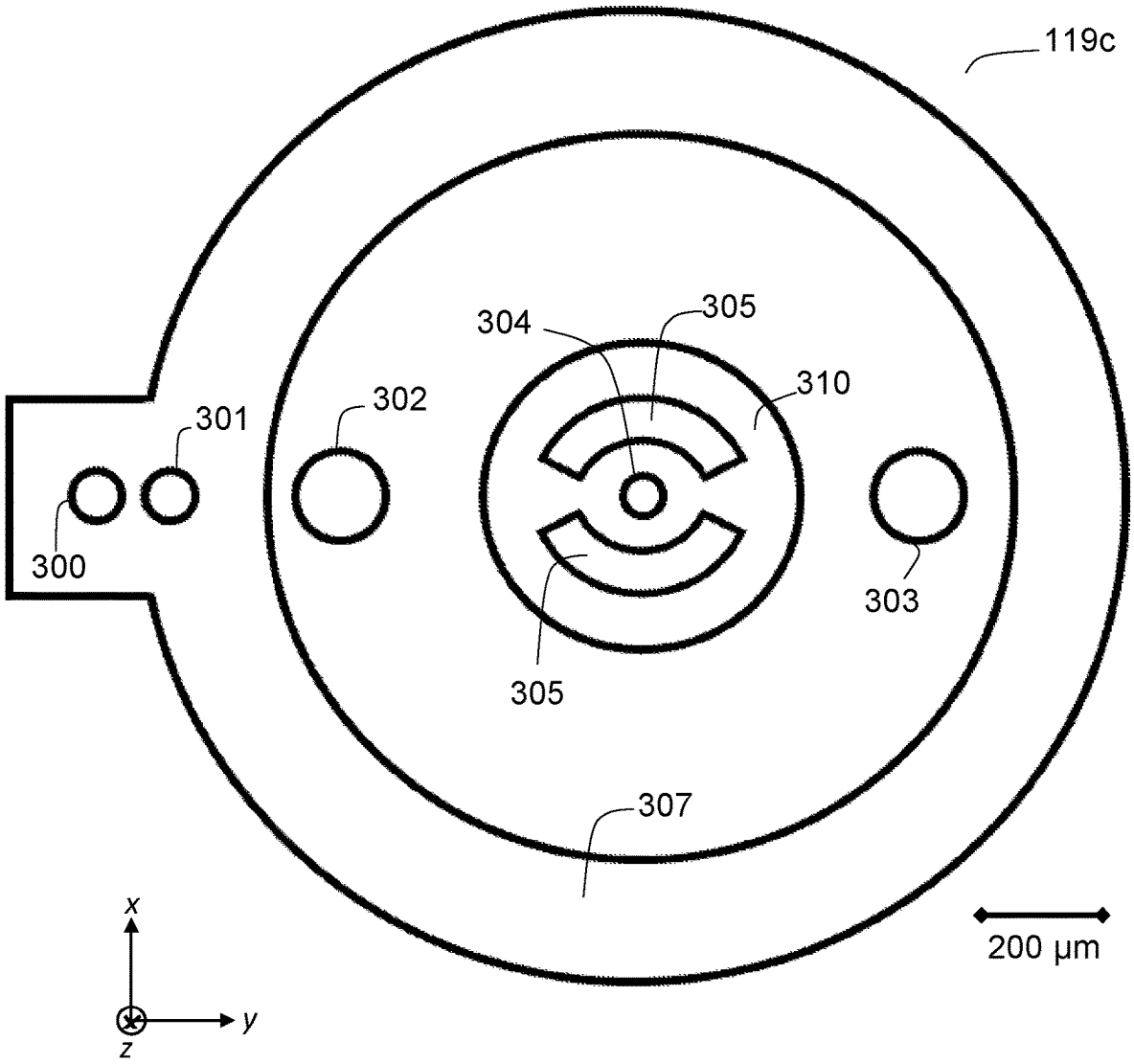

FIG. 6

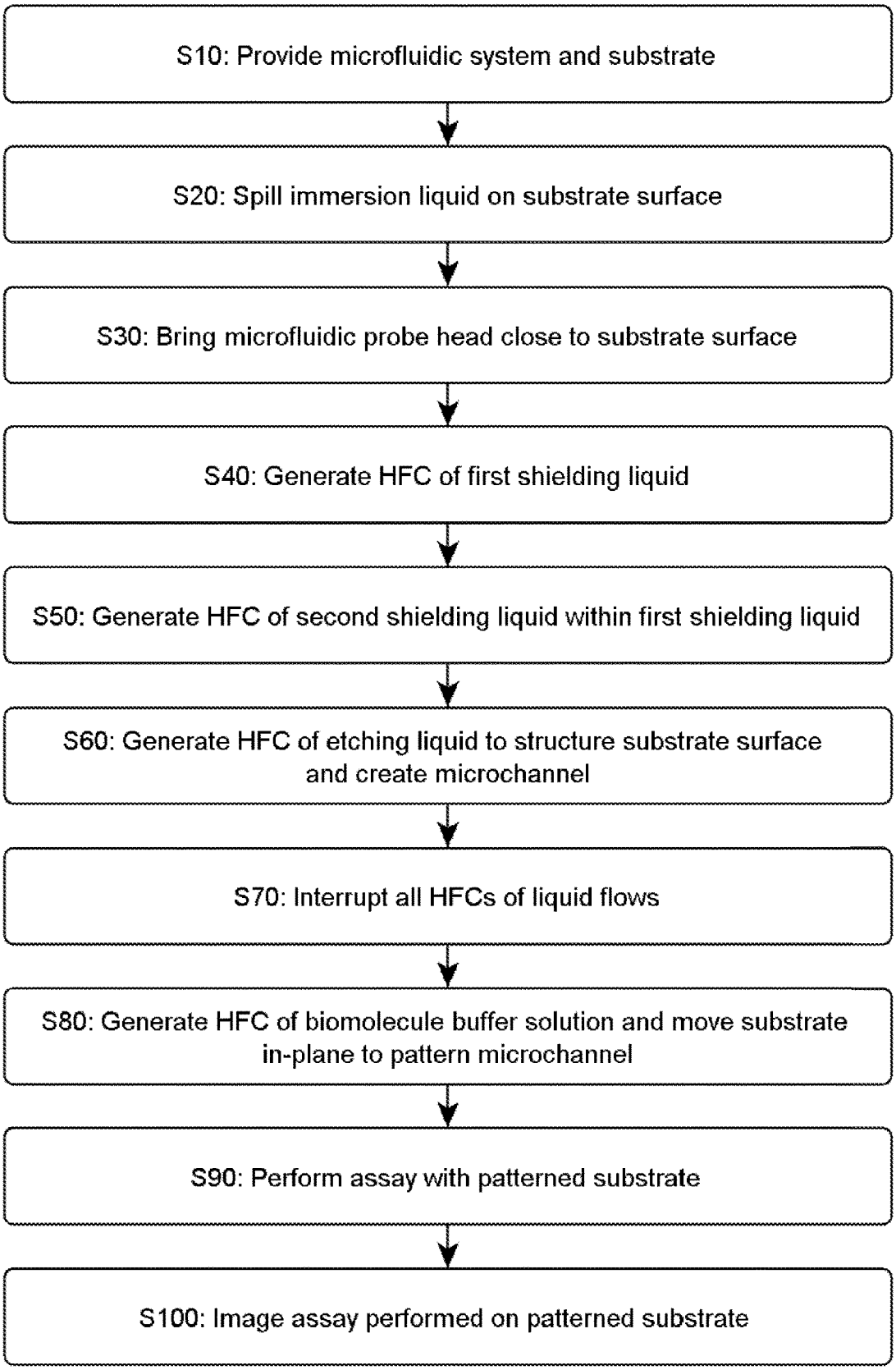
S10: Provide microfluidic system and substrate
S20: Spill immersion liquid on substrate surface
S30: Bring microfluidic probe head close to substrate surface
S40: Generate HFC of first shielding liquid
S50: Generate HFC of second shielding liquid within first shielding liquid
S60: Generate HFC of etching liquid to structure substrate surface and create microchannel
S70: Interrupt all HFCs of liquid flows
S80: Generate HFC of biomolecule buffer solution and move substrate in-plane to pattern microchannel
S90: Perform assay with patterned substrate
S100: Image assay performed on patterned substrate

FIG. 7

SUBTRACTIVE MICROFABRICATION AND FUNCTIONALIZATION OF SUBSTRATES BY HYDRODYNAMIC FLOW CONFINEMENTS

BACKGROUND

The invention relates in general to microfluidic techniques and, in particular, to methods and systems for patterning a substrate using microfluidic probes. It notably concerns methods of patterning a substrate by subtractive microfabrication followed by a selective functionalization of the substrate, e.g., with biomolecules.

Microfluidics deals with the precise control and manipulation of small volumes of fluids. Typically, such volumes are in the sub-milliliter range and are constrained to channels having heights and widths on the order of the micrometer. Prominent features of microfluidics originate from the peculiar behavior that liquids exhibit at the micrometer length scale. Flow of liquids in microfluidics is typically laminar. Volumes well below one nanoliter can be reached by fabricating structures with lateral dimensions in the micrometer range. Microfluidic devices generally refer to microfabricated devices, which are used for pumping, sampling, mixing, analyzing and dosing liquids.

Many microfluidic devices have user chip interfaces and closed flow paths. Closed flow paths facilitate the integration of functional elements (e.g., heaters, mixers, pumps, UV detector, valves, etc.) into one device while minimizing problems related to leaks and evaporation.

Microfluidics has opened the door for applications in many areas of healthcare and life sciences, such as point-of-care diagnostics (POCDs), environmental analysis, and drug discovery. POCDs strongly benefit from microfluidic technologies due to the miniaturization of tests, which enhances portability and the integration of various functions into one diagnostic device. For instance, many lateral flow assay tests rely on microfluidic functions and microfabrication to increase their precision and multiplexing capabilities.

Biochips are miniaturized microfluidic devices that integrate biological components, which enable new capabilities and higher efficiencies in the analysis and control of biological samples. The use of biochips has been rapidly growing in the field of in vitro analysis both for research applications and for diagnostics. Their manufacturing remains bound to a sequence of traditional process steps, each having its own requirements and limitations. Microchannels and microstructures are often obtained through micro-milling, laser ablation, embossing, and/or mold injection methods. A major limitation of these methods is that they are performed in a dry environment, which is unsuitable for biological components such as antibodies, DNA probes, and cells. Thus, additional preparation procedures are required prior to bio-patterning the substrates. In addition, the bio-patterning steps require re-aligning the substrates. The bio-patterning is carried out using either inkjet spotting or contact application.

SUMMARY

According to a first aspect, the present invention is embodied as a method of patterning a substrate. The method makes use of a substrate and a microfluidic probe head. The surface of the substrate is covered by an immersion liquid and the probe head is positioned in proximity with the surface of the substrate, so as to immerse a processing surface of the probe head in the immersion liquid. Next, liquid flows are generated between the processing surface of the probe head and the surface of the substrate, via the probe head. The liquid flows generated include a flow of an etching liquid (e.g., an acid or solvent) and a flow of a processing liquid (e.g., a solution or suspension). Such flows are referred to an etching flow and a processing flow. The etching flow is hydrodynamically confined inside the immersion liquid to controllably structure the surface of the substrate. This causes to create a depression in the substrate, e.g., as a result of locally etching or dissolving the substrate. The processing flow is generated after having interrupted the etching flow. The processing flow generated causes to pattern the depression created with particles (e.g., biomolecules, or metal or polymer particles) contained in the processing liquid.

The proposed approach makes it possible to fabricate a substrate with functionalized microstructures within a same process, which allows a significant simplification of the fabrication process and gain in fabrication time. In particular, the present approach allows biofunctionalized chips to be obtained, opening new possibilities for biochip design. E.g., an assay may be performed right after the patterning step.

In preferred embodiments, the depression created is subsequently patterned by hydrodynamically confining the processing flow inside the immersion liquid, vis-à-vis the depression. I.e., an additional flow confinement is used to controllably deposit the particles (e.g., biomolecules) onto the depressions formed, to improve the selectivity of the patterning.

In embodiments, the method further comprises moving the substrate in a plane parallel to an average plane of the substrate while maintaining the hydrodynamically confined flow of etching liquid, to form a microchannel in the substrate.

Shielding liquids can be used to shield the immersion liquid from the etching liquid. In embodiments, the generated liquid flows further comprise a shielding flow of a shielding liquid. The shielding flow is generated prior to generating the etching flow. The shielding flow is hydrodynamically confined inside the immersion liquid. The etching flow is subsequently generated so as to be hydrodynamically confined within the shielding flow.

Preferably, a further shielding flow (referred to as a first shielding flow) is generated prior to generating the above shielding flow (referred to as a second shielding flow). The respective liquids are accordingly referred to as a first shielding liquid and a second shielding liquid. The first shielding flow is hydrodynamically confined inside the immersion liquid, whereby the etching flow is hydrodynamically confined within the second shielding flow, which is itself hydrodynamically confined within the first shielding flow. Nested confinements are accordingly generated, inwardly, to better preserve the immersion liquid.

For example, the substrate may comprise polystyrene and the etching liquid may comprise dichloromethane. In this case, the second shielding liquid preferably comprises ethanol. Each of the first shielding liquid and the immersion liquid may for instance comprise water.

In typical embodiments, each of the first shielding flow, the second shielding flow, the etching flow, and the processing flow, is generated by ejecting liquid via an aperture of the probe head and aspirating liquid between the processing surface of the probe head and the surface of the substrate. There, various parameters can be adjusted to optimize the hydrodynamic flow confinements. For instance, a ratio between flow rates of the ejected liquid and the aspirated liquid can be set between 1/3 to 1/20. Preferably, said flow

rates are, each, between 0.1 and 200 μl/min. Moreover, the probe head is preferably positioned so as for the processing surface to be at a distance of the exposed surface, wherein said distance is between 20 and 200 μm.

For example, the surface of the substrate may be controllably structured so as for one or each of an average depth and an average diameter of the created depression to be between 1 and 500 μm.

In embodiments, the method further comprises performing an assay with the patterned substrate, after having patterned the depression with the particles. The assay may possibly be imaged, thanks to an imaging system (e.g., comprising an inverted microscope).

In preferred embodiments, the processing surface of the probe head is structured so as to comprise at least two apertures, these including a first aperture for ejecting liquid toward the surface of the substrate and a second aperture for aspirating liquid between the processing surface of the probe head and the surface of the substrate. Preferably, the second aperture is curved, and the first aperture is at least partly surrounded by the second aperture, so as to optimize the evacuation of the etching liquid.

In fact, several sets of ejection/aspiration apertures may advantageously be relied on. E.g., the processing surface of the probe head may be structured so as to comprise at least four apertures, these including at least two apertures for ejecting liquid toward the surface of the substrate and at least two apertures for aspirating liquid between the processing surface of the probe head and the surface of the substrate. E.g., six or seven apertures may be formed on the processing surface.

According to another aspect, the invention is embodied as a microfluidic system. The system comprises a first motorized stage including a holder designed to receive a substrate. The first motorized stage is configured to move the substrate parallel to a reference plane, in operation. The system further includes a second motorized stage with a microfluidic probe head mounted thereon. The second motorized stage is configured to move the microfluidic probe head perpendicularly to the reference plane. Moreover, the system comprises a liquid driving system including liquid supplies connected to the probe head. The liquid supplies comprise an etching liquid supply and a processing liquid supply. The control unit is connected to the liquid driving system, the first motorized stage, and the second motorized stage. The control unit, the liquid driving system, and the probe head, are configured to sequentially generate flows of liquids (as supplied by the liquid supplies) between a processing surface of the probe head and the surface of the substrate and allow the generated flows to be hydrodynamically confined inside an immersion liquid covering the surface of the substrate, in operation.

Preferably, the liquid supplies additionally comprise a shielding liquid supply. In that case, the control unit, the liquid driving system, and the probe head are configured to generate nested flows of hydrodynamically confined liquids between the processing surface of the probe head and the surface of the substrate, in operation.

In preferred embodiments, the probe head has a processing surface that includes a liquid aspiration channel and a curved ejection channel, the latter comprising one or more air bubble traps, each of the traps including a set micropillars. The liquid aspiration channel is connected to the curved ejection channel on the processing surface, so as to allow air bubbles guided by the air bubble traps to be evacuated via the liquid aspiration channel, in operation.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and advantages of the present invention will become apparent from the following detailed description of illustrative embodiments thereof, which is to be read in connection with the accompanying drawings. The illustrations are for clarity in facilitating one skilled in the art in understanding the invention in conjunction with the detailed description. In the drawings:

FIG. **1** is a diagram schematically illustrating components of a microfluidic system according to embodiments;

FIGS. **2**A-**2**C are 2D cross-sectional views illustrating steps of structuring and functionalizing a substrate with a microfluidic probe head, where the substrate is structured via a hydrodynamic flow confinement (HFC) of an etching liquid and then patterned thanks to an HFC of a processing liquid that contains biomolecules, according to embodiments;

FIGS. **3**A-**3**D are 2-dimensional (2D) cross-sectional views illustrating steps of structuring and functionalizing a substrate with a microfluidic probe head, similar to FIGS. **2**A-**2**C, except that nested HFCs of liquids are involved, as in embodiments;

FIGS. **4** to **6** show processing surfaces of various microfluidic probe heads as used in embodiments. Note, FIG. **4** is a front view of the processing surface (i.e., the apex) of the microfluidic probe head seen in cross section in FIGS. **3**A-**3**C. FIG. **5** is a front view of the apex of a bilayer microfluidic chip in a "vertical" configuration, having apertures arranged at locations corresponding to locations of apertures seen in the cross-sectional views of FIGS. **3**A-**3**C; and

FIG. **7** is a flowchart illustrating high-level steps of a method of patterning a substrate, according to embodiments;

The accompanying drawings show simplified representations of devices or parts thereof, as involved in embodiments. Technical features depicted in the drawings are not necessarily to scale. Similar or functionally similar elements in the figures have been allocated the same numeral references, unless otherwise indicated.

Microfluidic systems and methods embodying the present invention will now be described, by way of non-limiting examples.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The following description is structured as follows. General embodiments and high-level variants are described in section **1**. Section **2** addresses more specific embodiments and technical implementation details. Note, the present method and its variants are collectively referred to as "the present methods". All references Sn refer to methods steps of the flowchart of FIG. **7**, while numeral references pertain to physical parts or components of the system **1** shown in FIG. **1**, or to elements involved in embodiments.

1. General Embodiments and High-Level Variants

In reference to FIGS. **1**, **2**A-**2**C, **3**A-**3**D, and **7**, an aspect of the invention is first described, which concerns a method of patterning a substrate.

The method essentially involves S**10** a substrate **118** and a microfluidic probe head **119**, **119***a* (or probe head for

short). An immersion liquid **106** is spilled over an exposed surface of the substrate **118**, so as for the immersion liquid **106** to cover S**20** the surface. Next, the probe head **119**, **119***a* is positioned S**30** in proximity with the surface of the substrate. Note, the probe head **119**, **119***a* may be positioned prior to covering the substrate with the immersion liquid **106**. In all cases, the probe head **119**, **119***a* is brought sufficiently close to the substrate **118** so that, eventually, a processing surface (or apex) of the probe head is immersed in the immersion liquid **106**.

Next, liquid flows are generated S**40**-S**80** between the processing surface of the probe head and the surface of the substrate **118**, via the probe head **119**, **119***a*. The liquid flows generated include a flow **108** of an etching liquid and a flow **121** of a processing liquid. The latter comprises particles to be deposited on the surface of the substrate **118**. In the following, the flows **108** and **121** are referred to as the "etching flow" and the "processing flow", respectively.

The etching flow **108** is generated so as to be hydrodynamically confined S**60** inside the immersion liquid **106**. A hydrodynamic flow confinement (HFC) is accordingly achieved, which allows the surface of the substrate **118** to be controllably structured. This is achieved by subtraction, thanks to the etching liquid **108**, which, e.g., locally etches or dissolves the substrate. One or more depressions **120** (i.e., cavities) are accordingly created in the substrate **118**.

The flows **108**, **121** are sequentially generated, though not concomitantly. Rather, the processing flow **121** is generated S**80** after having interrupted the etching flow **108**. The flow **121** is applied so as to pattern S**80** the depression **120** created at step S**60** with particles **122** contained in the processing liquid. That is, particles from the processing liquid are deposited onto the depressions, where they may possibly be immobilized. After depositing the particles **122**, the substrate **118** may possibly be covered with a lid (e.g., a polymer layer, not shown), if necessary.

Additional liquid flows may possibly be sequentially generated, if needed. For instance, the etching flow **108** may be generated concomitantly with one or more additional liquid flows **107**, **109**, i.e., shielding flows, as described later in reference to preferred embodiments.

All such liquid flows are generated thanks to conduits or channels provided in the probe head **119**, **119***a*, where such conduits lead to respective apertures **100**-**105** formed on the processing surface of the probe head **119**, **119***a*. The processing surface, or apex, of the probe head is the surface that faces the substrate, in operation, i.e., the surface through which liquids are ejected and aspirated. The probe head may further comprise mesas and other structures, such as posts and pillars. Various types of microfluidic heads can be contemplated, as discussed later in detail.

Note, the terminology "etching liquid" should be understood broadly. E.g., the etching liquid **108** may also be regarded as a milling liquid. I.e., applying this liquid causes to locally mill, grind, chemically decompose, or otherwise subtractively alter a superficial thickness of the substrate **118**. The corresponding flow **108** is confined S**60** laterally inside the immersion liquid **106**, below the processing surface. Note, as evoked earlier, the etching liquid may only be indirectly confined in the immersion liquid **106**, inasmuch as one or more shielding flows may be concomitantly generated, so as to shield the immersion liquid from the etching flow, in a nested fashion. That is, the etching liquid **108** may possibly be confined in one or more shielding flows **107**, **109**, themselves confined in the immersion liquid **106**, in a nested fashion.

Thanks to the HFC of the etching liquid **108** applied, one or more depressions (i.e., cavities) are formed within a superficial thickness of the substrate **118**, in a controlled manner. I.e., the parameters controlling the HFC of the etching liquid **108** also control the resulting depression **120**. The substrate **118** may further be controllably moved S**80**, in-plane, while maintaining the etching flow **108**, such that a microchannel can be created, if necessary. That is, the substrate **118** may possibly be moved S**80** in a plane parallel to the average plane of the substrate (e.g., using a motorized stage) while maintaining the HFC **108** of etching liquid, to form a microchannel **120** (or another microstructure) in the substrate **118**. Therefore, the resulting substrate **118** can itself be regarded as a structured layer of a microfluidic device.

The proposed approach makes it possible to fabricate a substrate with functionalized microstructures, all within a same process. This allows a significant simplification of the fabrication process and gain in fabrication time. In particular, the present approach allows biofunctionalized chips to be obtained, opening new possibilities for biochip design.

Furthermore, the surface quality of the etched surface is optically clear, which is extremely useful for fluorescent-based detection and analysis. The proposed approach may find applications not only in the fields of diagnostics and analysis but also in tissue engineering, for example.

Advantageously, an assay may advantageously be performed S**90** with the substrate **118**, after having patterned S**80** the depression(s) **120** with particles **122**, as assumed in the flowchart of FIG. **7**. In fact, the assay may be performed right after the patterning step S**80**. In addition, the substrate **118** may possibly be imaged S**100** (e.g., by fluorescence imaging) while performing the assay, e.g., thanks to an imaging system **117** below the motorized stage **117**, as assumed in FIG. **1**.

The created depressions **120** are preferably patterned S**80** by hydrodynamically confining the processing flow **121** inside the immersion liquid **106**, vis-à-vis the depression **120**. I.e., given that the setup is designed to allow HFCs to be obtained, an additional HFC can advantageously be relied on to allow particles **122** (e.g., biomolecules) to be controllably deposited in the depressions **120** formed. I.e., this additional HFC allows a selective deposition (patterning) of the particles

In variants, the particles **122** may be deposited by merely ejecting the processing liquid **121**, without aspirating it (no HFC is needed in that case). Note, in that respect, that a bilayer substrate **118** may possibly be used, in which the top layer is decomposed upon applying the etching HFC **108**. This results in locally exposing the lower layer of the substrate, to which particles **122** subsequently adhere upon applying the processing flow **121**. However, the particles may not adhere to unetched portions of the top layer, such that a selective patterning can also be achieved. However, it will likely be simpler to rely on an additional HFC to pattern the depressions.

The processing liquid **121** is preferably a solution, i.e., a liquid mixture in which the particles (the solute) are uniformly distributed within a solvent. In less likely variants, the processing liquid may also be a liquid suspension.

Note, the term "particles" should be understood in a broad sense, inasmuch as such particles may comprise or consist of biomolecules, metal particles, or polymer particles, for example. Thus, various types of particles can be contemplated, which may have various properties and dimensions. Still, the term "particles" refer to well-identified particles, i.e., particles that can be unambiguously characterized, e.g.,

optically, after the patterning step S**80**. In addition, such particles denote particle that one intentionally wants to deposit on the surface of the substrate, as opposed to impurities, debris, or dust particles that may accidentally come to adhere to this surface.

In preferred embodiments, the particles **122** are biomolecules, as assumed in FIGS. **2**A-**2**C and **3**A-**3**D. In particular, different types of biomolecules may be contained in the solution **121**. The biomolecules come to contact the surface of the substrate **118** and are immobilized thereon. Such embodiments allow microstructures to be milled and biopatterned in a single process; they can notably be used to prototype and fabricate of biochips for microfluidic biomedical analyses. Controllably depositing biomolecules in the depression **120** via an HFC allows a clean functionalization of the substrate **118** to be achieved, such that an assay may possibly be performed S**90** short after the deposition S**80**.

As noted earlier, the generated liquid flows may further include one or more shielding flows **107**, **109** of shielding liquids. The shielding flows **107**, **109** are generated S**40**, S**50** prior to generating the etching flow **108**. The flows **107**, **109** are hydrodynamically confined inside the immersion liquid **106**, e.g., in a nested fashion. The etching flow **108** is subsequently generated S**60** so as to be hydrodynamically confined within a shielding flow **109**, as depicted in FIG. **3**C. Shielding liquids can advantageously be used to shield the immersion liquid **106** from the etching liquid **108**. In variants, similar, intermediate liquid flows **107**, **109** can be used to enable or promote a chemical reaction, for example.

In particular, two shielding flows **107**, **109** may be sequentially generated, in a nested fashion, as illustrated in FIGS. **3**A-**3**B. That is, a first shielding flow **107** is generated at step S**40** (FIG. **3**A) prior to generating a second shielding flow **109**, step S**50** (FIG. **3**B). Thus, the flows **107**, **109**, and **108**, are sequentially (yet concomitantly) generated, see FIGS. **3**A-**3**C. As a result, the first shielding flow **107** is hydrodynamically confined inside the immersion liquid **106**, while the etching flow **108** is hydrodynamically confined within the second shielding flow **109**, which is itself hydrodynamically confined within the first shielding flow **107**. In other words, the successive flows are generated in a nested fashion, inwardly. Note, all flow confinements refer to liquid flows that are laterally confined in the plane (x, y) within another liquid flow or static liquid. A vertical confinement is otherwise ensured, between the processing surface of the probe head and the top surface of the substrate **118**.

The substrate **118** may for example comprise polystyrene. In that case, the etching liquid **108** may advantageously comprise dichloromethane. The second shielding liquid **109** may for instance comprises ethanol, while each of the first shielding liquid **107** and the immersion liquid **106** may be an aqueous solution, i.e., water, or any bio-friendly liquid. In variants, the substrate **118** may comprise any suitable synthetic polymers or hydrogels and other acids or solvents may be used as etching liquid **108** in place of dichloromethane. For example, an ethylenediaminetetraacetic acid may be used as etching liquid for processing ionically crosslinked hydrogels (e.g., alginate, hyaluronic acid). As another example, potassium hydroxide or hydrofluoric acid can be used to etch metal oxides. For completeness, various immersion liquids can be relied on, and a range of shielding solutions **107**, **109** are available, which can match such immersion liquids. For instance, a wide array of polar and non-polar solvents can be used to ensure matching miscibility, prevent surface tension-related instabilities, and unwanted chemical interactions (e.g., ethanol, acetone, water, and chloroform).

Besides, various types of microfluidic probe heads **119**, **119***a-d* can be contemplated, as illustrated in FIGS. **2**A-**6**. Typically, each of the flows **107**, **109**, **108**, and **121**, is generated by ejecting liquid via an aperture of the probe head and aspirating liquid between the processing surface of the probe head and the surface of the substrate **118**. Distinct sets of pairs of apertured may be relied on, as assumed in FIGS. **3**A-**6** and contrary to FIGS. **2**A-**2**B.

Various design parameters can be optimized, either independently or jointly, in order to optimize the liquid flows **107**-**109** and, in particular, to obtain optimal HFCs. To start with, the ratio between the flow rates of the ejected liquid and the aspirated liquid is preferably set between 1/3 to 1/20. The flow rates of the ejected liquid and the aspirated liquid will typically be, each, between 0.1 and 200 μl/min. The probe head can be connected to suitable pumping means **113**, to generate the desired flow rates, as known per se. For completeness, one may ideally position S**30** the probe head **119** such that a distance between the processing surface of the head and the exposed surface is between 20 and 500 μm. Larger distances may need be maintained, should the substrate surface be wavy (i.e., non-planar).

In practice, imposing such experimental parameters allows the surface of the substrate **118** to be controllably structured S**60**. The average dimension (depth and/or diameter) of the resulting depression(s) **120** will typically be between 1 and 500 μm. The depth is measured perpendicularly to an average plane of the substrate, i.e., along direction z in the accompanying drawings. The diameter of the created depression(s) **120** is measured in-plane, i.e., in the plane (x, y). And, as noted earlier, the substrate may possibly be moved in any direction in the plane (x, y), thanks to the motorized stage **116**, see FIG. **1**. Therefore, microchannels of any length (typically on the order of the centimeter) and shape (straight, bent, spiral, etc.) can potentially be etched/milled on the substrate **118**, thanks to the applied flow **108**.

In the examples of FIGS. **2**A, **2**B, the processing surface of the probe head **119** is structured so as to comprise two apertures **104**, **105** only. The apertures are formed by respective conduits extending through the probe head **119**. They include a first aperture **104** for ejecting liquids **108**, **121** toward the surface of the substrate **118**. The corresponding conduit is connected to a liquid driving system **113** (see FIG. **1**). In this example, the liquid driving system **113** is assumed to suitably switch liquid supplies provided to the conduit leading to aperture **104**, whereby distinct liquids can sequentially be ejected via the same aperture **104**. The second aperture **105** is for aspirating liquid **106**, **108**, **121** between the processing surface of the probe head **119** and the surface of the substrate **118**. This aperture **105** is defined by a corresponding conduit, assumed to be connected to aspiration means (not shown) forming part of the liquid driving system **113**. The system **113** may comprise usual equipment, such as, e.g., tubing ports, valves, pressure means, so as to allow HFCs of the liquids to be obtained. FIG. **2**C shows the resulting substrate **118**, once structured (a depression **120** is formed) and functionalized (biomolecules **122** are immobilized on the substrate **118** at the level of the depression **120**).

In the examples of FIGS. **3**A-**3**D, the processing surface of the probe head **119***a* is structured so as to comprise six apertures **100**-**105**. As in the example of FIGS. **2**A-**2**B, the aperture **104** is meant for ejecting liquids **108**, **121** toward the surface of the substrate **118**, while the aperture **105** is for aspirating liquid between the processing surface of the probe

head **119** and the surface of the substrate **118**, see FIGS. **3**C, **3**D. Additional apertures **100**, **101**, **102**, **103** are provided, in order to allow additional liquid flows to be formed under the processing surface.

In embodiments such as illustrated in FIGS. **3**A-**6**, the processing surface of the probe head **119** is structured to comprise at least four apertures (e.g., six or seven apertures, or more), including at least two apertures for ejecting liquid and at least two apertures for aspirating liquid. In the example of FIGS. **3**A-**3**D, the processing surface of the probe head **119***a* comprises six apertures **100**-**105**, in order to allow nested confinements of three liquid flows **108**, **109**, **107**, see FIG. **3**C. As explained earlier, the corresponding HFCs are sequentially generated, inwardly (compare FIGS. **3**A, **3**B, and **3**C).

A front view of the corresponding processing surface is shown in FIG. **4**. I.e., the processing surface is viewed from below (i.e., from the substrate **118**). As seen in FIG. **4**, the aspiration aperture **105** is curved and the aperture **104** is partly surrounded by the aperture **105** in this example. In variants, two or more curved aspiration apertures **305** may be relied on, whereby the ejection aperture **304** is partly surrounded by several aspiration apertures **305** in that case, see FIG. **6**.

In other variants, the probe head **119***b* is configured as a two-layer device, see FIG. **5**. In this example, the top-layer **119***b***1** is structured to form conduits, i.e., channels are grooved on a surface of the layer **119***b***1**, and closed by the bottom layer **119***b***2**, leading to apertures **100***v*, **101***v*, **102***v*, **103***v*, **104***v*, and **105***v*. The concept of the probe head shown in FIG. **5** is often referred to as a "vertical probe head". Posts **111***v* protrude from the processing surface, at the level of the bottom layer **119***b***2** in this example, to ensure a minimal gap between the processing surface and the processed surface of the substrate **118**.

In the examples of FIGS. **4**, **5**, and **6**, the apertures provided on the processing surface of the probe heads **119***a*, **119***b*, **119***c* are generally aligned. I.e., their projections in a plane (x, y) parallel to the processed surface of the substrate **118** is aligned. However, more sophisticated designs can be contemplated.

FIG. **6** shows another example of processing surface design; it shows the front side (apex) of a microfluidic chip **119***c*, where the inner set of apertures **304**, **305**, set on the central mesa **310**, are used to create the milling confinement. The larger circular apertures **302**, **303** set on the apex between the inner **310** and outer mesa **307** create the shielding confinement. The leftmost set of apertures **300**, **301** set on the outer mesa **307** are used to create the bio-patterning confinement. The scale bar is 200 μm long.

All the microfluidic probe head designs discussed herein have been fabricated and tested by the Inventors. For example, the microfluidic probe head **119***c* has been used for integrated mechanical structuring and biofunctionalization of microfluidic channels on substrates, enabling rapid prototyping of biochips. Such a probe head can be used to create HFCs to localize the interaction between a fluid and a substrate to a microscale region. By confining organic solvents and scanning with the probe head **119***c* over the substrate surface, microchannels as deep as 90 μm are fluidically milled. Subsequently, by switching the confined fluid from a milling solution to a patterning solution, biomolecules are immobilized on the surface of the microchannel. For example, a biotin functionalized biochip can be fabricated in less than 5 min out of a polystyrene slide. Its functionalities were validated by implementing on-chip electrokinetic sample focusing, pressure driven flow, and a surface-based reaction.

In general, the probe heads **119**, **119***a*-*d* are designed so to have conduits (e.g., microconduits) and/or channels (e.g., microchannels), leading to respective apertures, which may have any suitable shape (e.g., rounded, square, channel-like). In the present context, a microchannel can be formed as a groove on a main surface of a layer of the probe head, or be drilled or milled across such a layer, so as to extend parallel or transversely to this layer. This layer is for example a substrate, or any layer that is sufficiently thick to provide mechanical stability to the probe head, although mechanical stability may be provided by means of an additional layer of component of the probe head. The layer on which the microstructures are patterned may typically be an essentially planar object, notwithstanding a possible structuring. I.e., this layer may include various structures formed thereon, in particular microstructures, such as mesas or posts.

Preferably, a characteristic depth or diameter of the conduits or channels of the probe heads is in the micrometer-length range, i.e., between 1 μm and 500 μm. In fact, the dimensions of such conduits or channels should be suited for handling and working with the liquids involved (starting with the processing liquids), in terms of viscosity, density, and any other interface requirements. For instance, wider channels may be needed where the probe head interfaces with capillaries (tubings) and/or for handling viscous polymers, whereas the probe head may exhibit narrower channels closer to the apex and/or for handling low viscosity organic solvents, for example. Still, some particular structures of the probe heads may be in the nanoscale range or in the millimeter range, the probe heads as a whole typically being in the centimeter range.

Referring back to FIG. **1**, another aspect of the invention is now described, which concerns a microfluidic system **1**. Several aspects of this system have been explicitly or implicitly described in reference to the present methods. Such aspects are, accordingly, only briefly discussed in the following.

Basically, the system **1** comprises a first motorized stage **116**, a second motorized stage **115**, a liquid driving system **113**, and a control unit **112**. It preferably has a modular design.

The first motorized stage **116** includes a holder, which is designed to receive a substrate **118**. In general, the first motorized stage **116** is configured to move the substrate parallel to a reference plane, i.e., parallel to the plane (x, y), in operation.

The second motorized stage **115** comprises a microfluidic probe head **119** mounted thereon. In general, the second motorized stage is configured to allow the microfluidic probe head **119** to be moved perpendicularly to the reference plane, i.e., along direction z, so to bring the probe head **119** closer to the substrate **118**, in operation. If necessary, the stage **115** may be rotated/tilted at any angle, so as to be able to adjust the liquid ejection angle.

Preferably, the motorized stages **115**, **116** are chosen so as to allow variable speed control for the sample holder and the microfluidic probe head (typically between 10 μm/s and 5 mm/s). Such motorized stages are known per se; as a whole, the elements **115**, **116**, **118**, **119** can be compared to a non-contact scanning system. Each motorized stage typically includes a microcontroller, a stepper motor driver (with microstepping) and a stepper motor. High-end, mid-range, or off-the shelf the motorized stages can be contemplated.

The liquid driving system **113** includes liquid supplies (not shown) that are connected to the probe head **119**, e.g., via tubing **114** and tubing ports (not shown), as schematically depicted in FIG. **1**. Such liquid driving systems are known per se; they may include motorized elements, controllers, pumps, etc. The liquid supplies notably comprise an etching liquid supply and a processing liquid supply. They may additionally comprise supplies for the immersion liquid and the shielding liquids, as needed in applications.

The control unit (or control system) **112** may typically comprise a mini computer (e.g., Raspberry Pi Zero), as well as controller electronics. The unit **112** is adequately connected to each of the liquid driving system **113**, the first motorized stage **116**, and the second motorized stage **115**, to allow the system **1** to be operated. In particular, the control unit **112**, the liquid driving system **113**, and the probe head **119** are together configured to sequentially generate flows of liquids supplied by the liquid supplies. Such flows are formed between the processing surface of the probe head **119** and the surface of the substrate **118**. The generated flows can be hydrodynamically confined inside an immersion liquid **106** covering the surface of the substrate **118**, as discussed earlier in reference to the present methods.

In embodiments, the liquid supplies additionally comprise a shielding liquid supply. In that case, the control unit **112**, the liquid driving system **113**, and the probe head **119**, may be configured to generate nested flows of hydrodynamically confined liquids between the processing surface of the probe head **119** and the surface of the substrate **118**, in operation of the system **1**.

Consistently with the system **1** described above, the present methods may further comprise, prior to generating the liquid flows, connecting the probe head to liquid supplies of the liquid driving system **113**, mounting the substrate **118** onto the motorized stage **116**, and mounting the probe head to the motorized stage **115**.

The above embodiments have been succinctly described in reference to the accompanying drawings and may accommodate a number of variants. Several combinations of the above features may be contemplated. Examples are given in the next section.

2. Specific Embodiments and Technical Implementation Details

2.1 First Example of Particularly Preferred Embodiment (FIGS. **1** and **2**A-**2**C)

As depicted in FIGS. **1**, and **2**A-**2**C, a preferred approach for processing the surface of the substrate **118** in a wet environment utilizes a microfluidic probe head **119** and a non-contact scanning system **115**, **116** (FIG. **1**). This approach combines hydrodynamic confinement of liquids with precision motion control. The working principle of the microfluidic probe head **119** is depicted in FIG. **2**A-**2**B. In this example, the probe is a microfluidic chip (similar to that of FIG. **5**), which includes two microchannels leading to the tip ('apex') of the head. The apex is submerged within the immersion liquid **106** (e.g., water, buffers, cell medium) in proximity with the surface of the substrate **118**. The aperture **104** and the corresponding channel are suitably connected to liquid supplies to inject liquid. The other aperture **105** and the corresponding channel are connected to pressuring means (allowing negative pressures) to aspirate liquid at a much higher flow rate than used to inject liquid. Thus, a continuous supply of immersion liquid **106** is needed to keep the substrate and the apex immersed. Thanks to the flow rate differential achieved, an HFC can be formed below the apex. Particles (e.g., chemicals) contained within the HFC **108** make contact with the surface of the substrate **118** over a well-defined footprint, limiting the interaction to controlled length scales ranging from tens of micrometers to several centimeters.

Using the microfluidic probe head **119** for confining an etching liquid **108** (compatible with the surface of the substrate **118**) results in a highly localized fluidic milling of the surface of the substrate **118**, in accordance with the confinement footprint. The same set of injection and aspiration channels (or an additional adjacent set, as in FIGS. **3**A-**3**D) is then used to deliver a confined stream **121** of particles (e.g., biomolecules) **122** to the milled surface **120**, so as to pattern the depression **120** formed.

The probe head **119** can be mounted in a setup as depicted in FIG. **1**. The substrate **118** is set on a motorized (x, y) stage **116** atop an imaging system **117**, which may notably include an inverted microscope. The microfluidic probe head **119** is set to a motorized z stage **115**. Control of flow rates is achieved using a set of syringe pumps forming part of a liquid driving system **113**. Coordination of the process is done through an adequately programmed controlled unit **112**.

2.2 Second Example of Particularly Preferred Embodiment (FIGS. **1**, **3**A-**3**D, and **4**)

A unique capability that is enabled by the flow confinements is the ability to utilize a wide variety of etching and/or milling liquids within a biofriendly aqueous environment. By adding additional injection and aspiration sites, one may incorporate outer "shielding" confinements between the milling confinement **108** and the immersion liquid **106**, as depicted in FIG. **3**C. This shielding confinement can notably be utilized to confine acids and/or solvents and prevent cross contamination.

This approach can be used for prototyping biochips, especially where there is a need for integration of biological elements with microstructures. This extends to the fabrication of microfluidic-based immunoassay biochips, patterning of DNA arrays in closed structures, and fabrication of single cell analysis devices.

In a further example, the system **1** comprises a liquid driving system **113** (for example including pressure, syringe, or electro-osmotic pumps), a series of capillaries **114**, connecters and switches, motorized stages **115**, **116**, where a microfluidic probe **119***a* is mounted on the top stage **115** in place of the probe **119** and a substrate **118** is received on a holder of the bottom stage **116**, a goniometer for controlling the location of the probe **119***a* with respect to the substrate **115**, **116**, and an imaging setup **117**. All of these elements are controlled through a user interface on the control system **112**.

As assumed in FIGS. **3**A-**3**D, the microfluidic probe **119***a* is initially aligned and placed in proximity with the substrate **118** at a distance of 20-200 μm within an aqueous environment **106** (immersion liquid) on top of the substrate **118**.

A set of apertures **100**-**105** are used to inject and aspirate liquids. In order to ensure suitable flow confinements of the liquids, a flow rate ratio of 1/3 to 1/20 (injection to aspiration) is maintained. Flow rates for injection and aspiration are in the range of 0.1-200 μl/min.

The milling process is initiated through the confinement of three different liquids in nested hierarchical confinements **107**, **108**, **109**, see FIG. **3**C. The confinements are initiated from the outermost liquid, inwardly. For example, one may

mill polystyrene using dichloromethane. A confined liquid (e.g., water) **107** shields the outermost immersion liquid **106** from inner liquids (solvents) to prevent diffusive contamination. This confinement **107** is achieved by injecting an etching liquid from the outer injection channel **100** (FIG. **3**A) and aspirating (essentially this liquid **107**) from the opposite aspiration channel **101**. The middle confinement (ethanol) **109** is then initiated, see FIG. **3**B.

The middle confinement **109** ensures that the upcoming inner confinement **108** (FIG. **3**C) does not interact with the outer flow confinement **107**. This confinement **109** is realized by injecting from the injection aperture **102** and aspirating from the aspiration aperture **103**. Finally, the inner confinement (dichloromethane) **108** is initiated, beginning the milling process as the dichloromethane comes in contact with the substrate **118**, see FIG. **3**C. This confinement is achieved by injecting from the injection aperture **104** and aspirating from the aperture **105**.

Once the inner flow confinement **108** is initiated, the milling process occurs as the solvent mills away the substrate **118**. The depth and cross section of the milled microchannel **120** can then be controlled and altered by changing the injection and aspiration rates as well as the scanning speed and distance between the probe **119***a* and the top surface of the substrate **118**. For example, the depth and width of the depression formed may range from 1 to 500 μm.

Scanning with the probe **119***a* over the substrate **118** is facilitated by the motorized stages **115**, **116**. Note, the shape, size, and in-plane arrangement of the injection **100**, **102**, **104** and aspiration apertures **101**, **103**, **105** may differ, depending on the desired application.

Once the milling step is completed, the inner confinement **108** is interrupted and the liquid supply is then switched (either on or off the microfluidic chip); it is preferably switched automatically by the system **113**, off the chip. An additional, inner confinement **121** is then started, FIG. **3**D, which may for example consist of a buffer containing biomolecules. The biomolecules are deposited and immobilized on the surface of the substrate **118**. Scanning the probe **119***a* over the surface of the milled channel **120** allows the channel **120** to be adequately patterned by biomolecules, i.e., a selective patterning is achieved.

One of the parameters enabling the above-mentioned method is the distance between the apertures and the surface of the substrate **118**. Higher volume confinements can easily be achieved by increasing the distance between the apertures and the substrate **118**.

The processing surface (or apex) may further comprise a mesa **110** (compare FIGS. **3**A and **4**), in order to optimize the middle confinement **109** and adequately shield the inner flow confinement **108**, inasmuch as the mesa **110** force the inner flow **108** to be closer to the substrate surface. Additional barriers **111** are provided to help separating the outer confinement **107** from the middle confinement **109**. The barriers cause the inner confinement **108** to essentially interact with the substrate **118** and the middle confinement **109**. The height of the mesa **110** and the barriers **111** is typically between 1 and 100 μm.

2.3 Example of Operation Flow

As illustrated in FIG. **7**, a preferred flow of operation is the following. Some of the numeral references further refer to FIGS. **1** and **3**A-**3**D. At step S**10**, a microfluidic system **1** and a substrate **118** are provided. The substrate **118** is mounted in a holder of the motorized stage **116** of the system **1**. At step S**20**, immersion liquid is spilled over the substrate surface, e.g., using the probe head or an additional liquid inlet. At step S**30**, the microfluidic probe head is brought in close proximity with the substrate surface. At step S**40**, an HFC of first shielding liquid is generated. At step S**50**, an HFC of a second shielding liquid is generated so as for the second HFC to be nested in the HFC of the first shielding liquid. At step S**60**, an HFC of etching liquid is generated to structure the substrate surface. The stage **116** concomitantly moves the substrate **118** to create a microchannel. At step S**70**, all HFCs are interrupted. At step S**80**, a new HFC of a biomolecule buffer solution is generated and the substrate in concomitantly moved in-plane to pattern the previously obtained microchannel. At step S**90**, an assay is performed using the patterned substrate. The assay is imaged using an imaging system **117** at step S**100**. I.e., steps S**90** and S**100** are concomitant.

In an embodiment, a method of patterning a substrate can be provided. There can be a substrate and a microfluidic probe head. The surface of the substrate is covered by an immersion liquid and the probe head is positioned in proximity with the surface of the substrate, so as to immerse a processing surface of the probe head in the immersion liquid. Next, liquid flows are generated between the processing surface of the probe head and the surface of the substrate, via the probe head. The liquid flows generated include an etching flow of an etching liquid (e.g., an acid or solvent) and a processing flow of a processing liquid (e.g., a solution or suspension). The etching flow is hydrodynamically confined inside the immersion liquid to controllably structure the surface of the substrate, which causes to create a depression in the substrate. The processing flow is generated after having interrupted the etching flow. The processing flow generated causes to pattern the depression created with particles (e.g., biomolecules) contained in the processing liquid. Related microfluidic systems can also be provided.

While the present invention has been described with reference to a limited number of embodiments, variants and the accompanying drawings, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the scope of the present invention. In particular, a feature (device-like or method-like) recited in a given embodiment, variant or shown in a drawing may be combined with or replace another feature in another embodiment, variant or drawing, without departing from the scope of the present invention. Various combinations of the features described in respect of any of the above embodiments or variants may accordingly be contemplated, that remain within the scope of the appended claims. In addition, many minor modifications may be made to adapt a particular situation or material to the teachings of the present invention without departing from its scope. Therefore, it is intended that the present invention not be limited to the particular embodiments disclosed, but that the present invention will include all embodiments falling within the scope of the appended claims. In addition, many other variants than explicitly touched above can be contemplated. For example, even though specific setups and methods have been described above, there are many ways to implement the present methods for various applications, e.g., using different microfluidic chip geometries. In order to attain different shapes of flow confinements and different levels of shielding and interfaces between the different flow confinements, a number of design features can be adjusted (e.g., injection and aspiration aperture locations, shapes, and sizes). Furthermore, various flow rates can be contemplated. Moreover, in

variants to biomolecules, the patterning process may involve liquids containing metal or polymer particles. The middle and outer confinements may serve to shield or prevent cross contamination. In variants, they may be leveraged to facilitate a chemical reaction between the solutions.

What is claimed is:

1. A method of patterning a substrate, the method comprising:

providing the substrate and a microfluidic probe head;

covering a surface of the substrate by an immersion liquid and positioning the probe head in proximity with the surface of the substrate to immerse a processing surface of the probe head in the immersion liquid; and

via the probe head, generating liquid flows between the processing surface of the probe head and the surface of the substrate, wherein the liquid flows generated include:

an etching flow of an etching liquid that is hydrodynamically confined inside the immersion liquid to controllably structure the surface of the substrate by creating a depression in the substrate; and

a processing flow of a processing liquid, wherein the processing flow is generated after having interrupted the etching flow, to pattern the depression created with particles contained in the processing liquid.

2. The method according to claim **1**, wherein

the depression created is patterned by hydrodynamically confining the processing flow inside the immersion liquid, vis-à-vis the depression.

3. The method according to claim **2**, wherein

said particles are biomolecules.

4. The method according to claim **1**, wherein the method further comprises

moving the substrate in a plane parallel to an average plane of the substrate while maintaining the hydrodynamically confined flow of etching liquid, to form a microchannel in the substrate.

5. The method according to claim **1**, wherein

the generated liquid flows further comprise a shielding flow of a shielding liquid, wherein the shielding flow is generated prior to generating the etching flow and is hydrodynamically confined inside the immersion liquid, and

the etching flow is subsequently generated so as to be hydrodynamically confined within the shielding flow.

6. The method according to claim **5**, wherein

said shielding flow is a second flow and said shielding liquid is a second shielding liquid, and

the generated liquid flows further comprise a first shielding flow of a first shielding liquid, wherein the first shielding flow is generated prior to generating the second shielding flow and is hydrodynamically confined inside the immersion liquid, whereby the etching flow is hydrodynamically confined within the second shielding flow, which is itself hydrodynamically confined within the first shielding flow.

7. The method according to claim **6**, wherein

the substrate comprises polystyrene and the etching liquid comprises dichloromethane.

8. The method according to claim **7**, wherein

the second shielding liquid comprises ethanol.

9. The method according to claim **8**, wherein

each of the first shielding liquid and the immersion liquid comprises water.

10. The method according to claim **6**, wherein

each of the first shielding flow, the second shielding flow, the etching flow, and the processing flow, is generated by ejecting liquid via an aperture of the probe head and aspirating liquid between the processing surface of the probe head and the surface of the substrate, and

a ratio between flow rates of the ejected liquid and the aspirated liquid is between 1/3 to 1/20.

11. The method according to claim **10**, wherein

said flow rates are, each, between 0.1 and 200 μl/min.

12. The method according to claim **10**, wherein

the probe head is positioned so as for the processing surface to be at a distance from the surface of the substrate, said distance being between 20 and 200 μm.

13. The method according to claim **1**, wherein

the surface of the substrate is controllably structured so as for one or each of an average depth, and

an average diameter of the created depression to be between 1 and 500 μm.

14. The method according to claim **1**, wherein the method further comprises,

after having patterned the depression with particles, performing an assay with the patterned substrate.

15. The method according to claim **1**, wherein

the processing surface of the probe head is structured so as to comprise at least two apertures, these including a first aperture for ejecting liquid toward the surface of the substrate and a second aperture for aspirating liquid between the processing surface of the probe head and the surface of the substrate.

16. The method according to claim **15**, wherein

the second aperture is curved and the first aperture is at least partly surrounded by the second aperture.

17. The method according to claim **15**, wherein

the processing surface of the probe head is structured so as to comprise at least four apertures, these including at least two apertures for ejecting liquid toward the surface of the substrate and at least two apertures for aspirating liquid between the processing surface of the probe head and the surface of the substrate.

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